

Evaluation of Bisphenol A (BPA) Exposures on Prostate Stem Cell Homeostasis and Prostate Cancer Risk in the NCTR-Sprague-Dawley Rat: An NIEHS/FDA CLARITY-BPA Consortium Study

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BACKGROUND: Previous work determined that early life exposure to low-dose Bisphenol A (BPA) increased rat prostate cancer risk with aging. Herein, we report on prostate-specific results from CLARITY-BPA (Consortium Linking Academic and Regulatory Insights on BPA Toxicity), which aims to resolve uncertainties regarding BPA toxicity.

OBJECTIVES: We sought to a) reassess whether a range of BPA exposures drives prostate pathology and/or alters prostatic susceptibility to hormonal carcinogenesis, and b) test whether chronic low-dose BPA targets prostate epithelial stem and progenitor cells.

METHODS: Sprague-Dawley rats were gavaged daily with vehicle, ethinyl estradiol (EE) or 2.5–25,000 µg BPA/kg-BW during development or chronically, and prostate pathology was assessed at one year. One developmentally exposed cohort was given testosterone plus estradiol (T+E) implants at day 90 to promote carcinogenesis with aging. Epithelial stem and progenitor cells were isolated by prostatesphere (PS) culture from dorso-lateral prostates (DLP) of rats continuously exposed for six months to 2.5–250 µg BPA/kg-BW. Gene expression was analyzed by quantitative real time reverse transcription polymerase chain reaction (qRT-PCR).

RESULTS: Exposure to BPA alone at any dose did not drive prostate pathology. However, rats treated with EE, 2.5, 250, or 25,000 µg BPA/kg-BW plus T+E showed greater severity of lateral prostate intraepithelial neoplasia (PIN), and DLP ductal adenocarcinoma multiplicity was markedly elevated in tumor-bearing rats exposed to 2.5 µg BPA/kg-BW. DLP stem cells, assessed by PS number, doubled with chronic EE and 2.5 µg BPA/kg-BW exposures. PS size, reflecting progenitor cell proliferation, was greater at 25 and 250 µg BPA doses, which also shifted lineage commitment toward basal progenitors while reducing luminal progenitor cells.

CONCLUSIONS: Together, these results confirm and extend previous evidence using a rat model and human prostate epithelial cells that low-dose BPA augments prostate cancer susceptibility and alters adult prostate stem cell homeostasis. Therefore, we propose that BPA exposures may contribute to the increased carcinogenic risk in humans that occurs with aging. <https://doi.org/10.1289/EHP3953>

Introduction

Bisphenol A (BPA) is a high-production chemical; >5 million tons are produced annually worldwide, leading to global distribution in effluent discharges, sewage, surface waters, sediments, soil, air, wildlife, and humans (Corrales et al. 2015). In addition to contact with the above sources, human BPA exposure occurs through migration from food cans, polycarbonate plastics, thermal paper, dental sealants, and other BPA-containing products during routine use. Despite rapid clearance within 6 h of uptake (Völkel et al. 2002), most humans have measurable BPA in their urine (Calafat et al. 2008), indicating chronic exposure. Estimates for human exposures are 0.01 to >5 µg/kg-BW/day for adults and 0.01 to 13 µg/kg-BW/day for children in westernized countries and higher exposures in Asia (Corrales et al. 2015; Covaci et al. 2015; Geens et al. 2012). Human fetal exposure is documented through cord blood (Gerona et al. 2013), maternal blood at delivery (Padmanabhan et al. 2008), fetal tissue, placental tissue, and amniotic fluid measurements (Vandenberg et al. 2010),

with concentrations ranging from 0.14 to >250 ng/g. Potential effects are further compounded by a decreased ability of the human fetus and newborns to metabolize BPA into nonestrogenic forms, such as BPA-glucuronide (Nahar et al. 2013).

BPA is a recognized endocrine-disrupting chemical (EDC), with actions mediated through multiple membrane and nuclear estrogen receptors (ER), as well as interactions with other receptor pathways (Acconcia et al. 2015). Extensive animal-based and human epidemiology studies over the past 20 y have identified numerous adverse health effects from BPA exposures that include neurological and behavioral changes (Gore et al. 2015), obesity (Carwile and Michels 2011), altered male and female reproductive processes (Hass et al. 2016; Wang et al. 2014), mammary gland tumors (Acevedo et al. 2013), and prostate diseases (Prins et al. 2017), among others (Chapin et al. 2008; Gore et al. 2015; vom Saal et al. 2007). Of particular note, many of these studies identify the developmental period as a time of heightened sensitivity to BPA exposures, with modifications that can last throughout life or predispose to adult-onset diseases.

Previous work from our laboratory has shown that although developmental exposure to BPA at environmentally relevant doses is not sufficient to drive prostate pathology on its own, it reprograms the rat prostate epigenome and increases susceptibility to estrogen-driven carcinogenesis with aging (Cheong et al. 2016; Ho et al. 2006; Prins et al. 2011; Tang et al. 2012; Wong et al. 2015). In addition, using a humanized prostate model made from normal human prostate stem and progenitor cells, similar results were found wherein low-dose *in vivo* BPA exposure increased susceptibility to estrogen carcinogenicity, implicating direct relevance of the rodent model to human disease (Prins et al. 2014). Most recently, our detailed dose–response study, which included internal free BPA and BPA-glucuronide (BPA-G) dosimetry, demonstrated a nonmonotonic response to brief neonatal BPA exposures

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in a rat prostate lobe-specific manner. Significantly more lateral prostate high-grade intraepithelial neoplasia (PIN) lesions, the precursor lesion to prostate cancer (PCa), as well as progression to adenocarcinoma were found in rats developmentally exposed to low-dose BPA (10 µg/kg-BW or lower) with testosterone plus estradiol (T + E) implants given in adulthood that doubled circulating estradiol (Prins et al. 2017). This finding is biologically relevant because estradiol levels increase in aging men (Vermeulen et al. 2002), and together with T, induce prostate cancer in rat and human epithelia (Bosland et al. 1995; Hu et al. 2011) and accelerate PCa progression (Chakravarty et al. 2014; Setlur et al. 2008). Further, estrogenic activity is amplified in metastatic PCa in humans (Montgomery et al. 2010). Therefore, we propose that a combination of developmental BPA exposure with rising adult estrogen levels may augment PCa risk in the population.

Due to concerns about potential adverse effects of BPA, regulatory agencies have undertaken risk analyses and set guidelines based on the No Observed Adverse Effect Level (NOAEL), below which no adverse effects are observed, to establish what would be considered safe levels for BPA exposure. The current U.S. reference dose for BPA, calculated from the NOAEL, is set at 50 µg BPA/kg-BW/day. This level was based on a chronic rat study by the National Toxicology Program (NTP) undertaken in 1982 (NTP, 1982) that found a NOAEL of 5 mg BPA/kg-BW/day (CASRN 80-05-7) as well as a 2002 three-generation reproductive toxicity study using chronic BPA exposures that similarly found a 5 mg BPA/kg-BW/day NOAEL (Tyl et al. 2002). However, in 2008, the NTP determined that there was “some concern for effects on brain, behaviors and prostate gland in fetuses, infants, and children at current human exposures to BPA,” based on detailed review of an accumulating body of evidence indicating adverse effects at levels substantially below the NOAEL used by the U.S. Environmental Protection Agency (EPA) (Chapin et al. 2008). Nonetheless, based on this NTP report and additional reviews of its own, the U.S. Food and Drug Administration (U.S. FDA) currently concludes that “an adequate margin of safety exists for BPA at current levels of exposure from food contact uses” (U.S. FDA 2014). More recently, the European Food Safety Authority (EFSA) lowered their safe level [tolerable daily intake (TDI)] from 50 to 4 µg/kg-BW/day based on emerging data that suggests harm at lower levels (Bolognesi et al. 2015). These and other agencies acknowledge the need for continued investigations and evaluation of emerging studies and will update their recommendations accordingly when new findings are made available.

Recognizing the need for both good laboratory practices (GLP)-compliant studies or utilization of internationally validated test guidelines along with detailed behavioral, cellular, and molecular research to resolve controversies and uncertainties regarding BPA toxicity, an interagency collaboration was initiated between the National Institutes of Environmental Health (NIEHS), the NIEHS-NTP, and the U.S. FDA. Together, they established the “Consortium Linking Academic and Regulatory Insights on BPA Toxicity” (CLARITY-BPA), which has two parallel components: a) Core GLP guideline-compliant studies using 1- and 2-y chronic BPA exposures conducted at the U.S. FDA National Center for Toxicological Research (NCTR) laboratories to evaluate standard toxicology endpoints, and b) Academic laboratory studies by 14 NIEHS-funded research teams that comprehensively examined tissues and organ systems across a range of BPA doses in mechanistic and molecular detail not examined in the standard Core study (Schug et al. 2013). Importantly, all animals for both components were handled and treated at the U.S. FDA-NCTR laboratories using a common protocol and study design. Further, all data collection and analyses in academic laboratory experiments were

conducted in a blinded manner to treatment groups. In brief, NCTR Sprague-Dawley rats were gavaged daily with vehicle, one of five BPA doses from 2.5 to 25,000 µg/kg-BW/day, or ethinyl estradiol as a positive estrogen control. Two time periods of exposure were examined: “continuous exposure” from gestation day 6 (G6) to the time of sacrifice, and “stop-dose” exposure from G6 to postnatal day (PND) 21 to examine developmental exposure alone. Tissues were collected by NCTR staff and shipped to the academic laboratories for analysis, following guidelines requested by the independent groups. Final conclusions regarding potential health effects and reference dose revision are to be derived from combined analysis of the Core and Academic study components (Schug et al. 2013).

The objectives of the present CLARITY-BPA studies were to a) examine whether developmental and/or chronic BPA exposures are sufficient to drive pathology in separate regions of the prostate gland in rats supplied by the U.S. FDA; b) test the hypothesis that early life BPA exposures increase susceptibility to later-life neoplasia and adenocarcinoma in response to elevated estradiol levels, as occurs in aging men; and c) assess whether chronic BPA exposures modify stem cell homeostasis within the dorsolateral prostate (DLP) lobes. First, we histologically examined all prostate lobes and the periurethral prostatic ducts for pathologic lesions in two cohorts of rats, one exposed continuously from implantation through adulthood and the other developmentally exposed from implantation through PND 21, the “stop-dose” BPA cohort. Although the Core guideline chronic 2-y studies by the U.S. FDA also examined prostate pathology, they did not examine the periurethral prostatic ducts, considered an essential component for hormonal carcinogenesis studies (Bosland et al. 1995). Additionally, a “stop-dose” BPA cohort treated with T + E starting at PND 90 was included in the present studies to reexamine the initiator potential of developmental BPA to enhance the prostatic carcinogenic effects of later-life estrogens.

The second component of the present studies sought to directly address whether the prostate stem cell pool is a direct target of BPA exposures in this rodent model, as we previously determined for human prostate stem cells (Prins et al. 2014) and human embryonic stem cells (Calderon-Gierszal and Prins 2015). Using normal human prostate cells from young organ donors, our prior studies identified estrogen receptors (ER α , ER β , and GPER) in the isolated human prostate stem and daughter progenitor cells and determined that, as estradiol does, BPA increased their proliferative capacity (Prins et al. 2014). Importantly, BPA and estradiol-17 β (E $_2$) possessed equimolar membrane-initiated signaling in these cells with rapid activation of Akt and Erk signaling cascades. Further, BPA altered the stem/progenitor cell transcriptome, in part through histone modifications of a class of noncoding RNAs, thus identifying an epigenetic basis for prostate progenitor cell reprogramming (Ho et al. 2015). Because the studies with human stem cells were largely conducted *in vitro*, we herein sought to examine whether chronic *in vivo* BPA exposures could likewise reprogram the stem and progenitor cell populations in the rat DLP.

As delineated above, rather than manifest toxic effects of BPA on prostate pathology that were the only examined endpoints in Core guideline studies, we hypothesized that subtle, but equally harmful effects may occur whereby exposures to BPA reprogram the prostate and augment its susceptibility to PCa development from later-life encounters, such as rising estrogens with aging. Further, we hypothesized that chronic BPA exposures would modify prostate stem and progenitor cell homeostasis and potentially their responsiveness to E $_2$, which together, may underpin increased PCa risk with aging.

Materials and Methods

Animal Husbandry and Treatments

This study is a component of the CLARITY-BPA program, and detailed descriptions of study design and animal handling have been previously described (Heindel et al. 2015). Portions applicable to the prostate studies are presented herein. All animals utilized in the present studies were born, housed and treated at the U.S. FDA-NCTR facility (Jefferson, AR), using guideline GLP conditions. At the time of treatment, all rats were given a unique numeric identifier assigned by NCTR staff such that all primary data collection and analysis were conducted blind to exposure type and dosage. At the time of necropsy, the prostatic complex was collected *en bloc*, and de-identified fresh or paraffin-embedded tissues were shipped to the University of Illinois at Chicago (UIC) for studies as described below. All animal use and procedures for this study were approved by the NCTR Laboratory Animal Care and Use Committee and conducted in an AAALAC-accredited U.S. FDA-NCTR facility. NCTR Sprague Dawley cesarean-derived rats (strain code 23) were obtained from the NCTR breeding colony. Throughout the study, animal rooms were maintained at $23 \pm 3^\circ\text{C}$ with a relative humidity of $50 \pm 20\%$ and 12-h light/dark cycle, and food and water were available *ad libitum*. The animal diet was soy- and alfalfa-free to minimize phytoestrogen content (5K96 verified casein diet 10 IF, round pellets, γ -irradiated). The vehicle solutions and other study materials were screened for BPA, including animal bedding, polysulfone cage leachates, silicone water bottle stoppers, and drinking water. None of these materials had BPA levels detectable above the average analytical blanks. BPA (CAS no. 80-05-7, TCI America Lot no. 111,909/AOHOK, air-milled) and ethinyl estradiol (EE; CAS no. 57-63-6, Sigma Lot no. 028K1411) were used for treatments. The vehicle used to deliver the BPA and EE was 0.3% aqueous carboxymethylcellulose (CMC). CMC was obtained from Sigma-Aldrich (catalog no. C5013, lot no. 048K0023).

Dosing and tissue preparation for histopathology studies. Histopathology of the prostatic complex was assessed at one year of age in 3 sets of male rats: Set 1, continuous dosing regimen of BPA daily from G6 to 1 y as well as negative (vehicle) and positive (EE) controls ($n = 8\text{--}10$ /treatment); Set 2, stop-dose regime of BPA daily from G6 to PND 21 with vehicle and EE controls ($n = 8\text{--}10$ /treatment); Set 3, stop-dose regime (BPA daily from G6 to PND 21 plus controls) with T+E capsules implanted at PND 90 in all rats. The animals were unevenly distributed among the treatments in Set 3 with $n = 19\text{--}20$ for vehicle and EE rats and $n = 5\text{--}15$ for BPA-treated rats. Timed pregnant rats were gavaged daily with vehicle, EE ($0.5 \mu\text{g/kg-BW}$), or BPA at 2.5, 25, 250, 2,500, or 25,000 $\mu\text{g/kg-BW}$ from G6 until labor. Starting on PND 1 (day of birth is PND 0), the pups were gavaged daily with the same treatments until one year of age (Set 1) or until PND 21 (Sets 2 and 3). At PND 90, rats assigned to Set 3 were given subcutaneous implants of Silastic capsules (Dow Corning; i.d. 1.98 mm, o.d. 3.18 mm) packed with crystalline testosterone (two 2-cm tubes) and estradiol-17 β (one 1-cm tube) (T + E) (Sigma Aldrich) via a right-flank incision under anesthesia. The T capsules maintain physiological testosterone levels, and the E capsules double the circulating estradiol levels, which is sufficient to promote prostate cancer in a rat model (Ofner et al. 1992; Bosland et al. 1995). Unlike our prior rat studies, the hormone implants were not replaced every 8 wk to ensure maintenance of hormone levels. Nonetheless, independent studies have noted that Silastic capsules implanted without replacement retain substantive amounts of T and E after one year (M.C. Bosland, unpublished observations). The prostatic/urethral complex plus the seminal vesicles and coagulating glands were excised *en masse* at one year of age from all treatment groups and placed in 10% neutral buffered formalin for 72 h prior to histologic processing.

Dosing and tissue collections for prostate stem/progenitor cell biology studies. Timed pregnant rats were gavaged daily with vehicle, BPA at 2.5, 25, or 250 $\mu\text{g/kg-BW}$, or EE ($0.5 \mu\text{g/kg-BW}$) as a positive estrogenic control from G6 until labor. Starting on PND 1, male pups ($n = 10$ /treatment group) were gavaged daily with the same treatments until six months of age (continuous dose arm). At 6 months, the prostatic complex was removed under aseptic conditions, placed in chilled dissecting medium (DMEM containing 10% FBS, antibiotic-antimycotic; Gibco, Inc.) and express-shipped overnight with ice packs to UIC for delivery at 0800 hours. Five shipments of tissues over a three-month period were undertaken to optimize the work flow at the FDA-NCTR and UIC labs. Although all tissues were de-identified for treatment group, prostate complexes from 2 rats/treatment group were sent as pairs to permit tissue pooling during subsequent stem cell isolation procedures (see below). The prostatic complex was dissected into separate lobes, and the DLP were used for subsequent stem/progenitor cell cultures. This procedure was based on prior studies that showed the dorsal and lateral lobes as the most estrogen- and BPA-sensitive (Prins 1992; Prins et al. 2011; Prins et al. 2017) and the DLP region having homology to the human prostate (Price 1963).

Histopathology Analysis

The fixed tissue was dissected for subsequent dehydration and embedded in three paraffin blocks per animal; Block 1 (paired seminal vesicles and coagulating gland, a.k.a. anterior prostate), Block 2 (ventral prostate), and Block 3 (remaining prostatic complex consisting of the lateral and dorsal lobes and the periurethral region). For Block 3, the lobes were cut in halves at right angles to the prostatic urethra and embedded together in paraffin to allow simultaneous examination of DLP lobes and ducts, including the periurethral area where carcinomas develop in T + E-treated rats (Bosland et al. 1995). Sections of four μm thickness were made and stained with hematoxylin and eosin (H&E). Six step sections were made from the dorsolateral complex at 250 μm intervals, allowing for the detection of the maximum number of microscopic-sized lesions (McCormick et al. 1998), and one section was made of the ventral prostate, which is not an estrogen target organ in this model. All prostate lobes and periurethral prostatic ducts were evaluated histopathologically in a blinded fashion by a single pathologist (M.C.B.), and the presence, type, and size of all lesions were scored using previously published criteria (Bosland et al. 1995; McCormick et al. 1998). This scoring included detection of inflammatory cell infiltration, reactive epithelial hyperplasia (in epithelium proximate to areas of inflammation), and prostatic intraepithelial neoplasia (PIN, a.k.a. dysplasia or atypical hyperplasia). For each, severity scores were assigned as follows: 0 (no lesions present) and 1, 2, 3, or 4 to indicate, respectively, minimal, slight, moderate, or marked severity. The presence and number of adenocarcinomas in the periurethral prostatic ducts were counted in every animal to determine the incidence and multiplicity of these tumors.

Primary Prostatosphere (PS) Culture from Rat Prostates

Effects of BPA on the DLP epithelial stem cells were assessed using 3-D PS culture ($n = 3\text{--}5$ /treatment) through three serial PS passages, which enriches tissue stem cells and permits propagation of their daughter progenitor cells. As such, PS were collected and analyzed 21 d after the last *in vivo* BPA exposure. DLPs were chopped and minced with microscissors in dissecting medium followed by digestion with collagenase (200 Unit/ml) for 2 h and Trypsin/EDTA (0.05%) for 5 min at 37°C . Mixed digested tissue suspension was passed through 18-G and 20-G needles five times, respectively. The cell mixture was filtered through a nylon mesh filter with a 40- μm pore size. Collected cells were pelleted

and resuspended in fresh ProstaLife™ Epithelial Cell Culture Medium (LifeLine Cell Technology). Prostate stem cells were isolated from the primary epithelial cells using a serum-free 3D Matrigel (Corning) culture system as previously described (Hu et al. 2011). In brief, 1×10^5 cells were resuspended in 1 ml 1:1 Matrigel/ProstaLife™ medium and plated around the rim in 6-well plates to allow the Matrigel to solidify, and then covered with 2 ml ProstaLife™ medium followed by incubation at 37°C in 5% CO₂ for 7 d to form first-generation (P1) PS. The culture media was replenished every 2–3 d. For serial passage, PS were dispersed into single cells by 1 U/ml dispase for 20 min and 0.05% trypsin digestion for 5 min and re-plated into 3D Matrigel culture to form second (P2) and third (P3) generations of PS, each cultured for 7 d. The third passage (P3) spheres were treated ± 1 nM E₂ for 7 d and harvested for assays below. Images of PS were photographed at 4 \times objective power using EVOS XL Cell Imaging System (ThermoFisher Scientific). PS number and size at day 7 were assessed using an automated digital image processing algorithm as previously described (Prins et al. 2014). PS forming efficiency was compared among different treatment groups using both PS number per rat and per 10,000 input cells. Samples remained de-identified until all data were uploaded to the NIEHS-managed database.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Gene expression of prostate stem/progenitor cells and differentiation markers were evaluated by real-time qRT-PCR. Total RNA was isolated from P3-PS using TRIzol® Reagent (Invitrogen) and cDNA was synthesized using iScript™ cDNA Synthesis kit (Bio-Rad) per manufacturer's instructions. PCR reactions with SsoAdvanced™ Universal SYBR® PCR Supermix (Bio-Rad) were carried out using CFX96 Real-Time System (Bio-Rad) (Hu et al. 2017). Primer sequences for all genes measured are provided in Table S1 and were synthesized at the UIC Genomics Facility Core. Real-time PCR reactions were carried out in duplicate with an initial denaturing at 95°C for 5 min followed by 40 cycles of denaturing at 95°C for 15 s and annealing/extension at 60°C for 1 min. Data was analyzed by $-\Delta\Delta C_t$ method. Expression levels of mRNA for each gene were normalized to house-keeping gene RPL19 levels.

Human Prostasphere Culture and Stem Cell Label-Retention Assay

Primary human prostate epithelial cells (PrEC) from disease-free organ donors were cultured in ProstaLife™ medium in the presence of 1 μ M BrdU (Sigma-Aldrich) for 10 d to label all dividing cells as previously described (Hu et al. 2017). PrEC were next transferred to 3D Matrigel culture for 5 d to permit BrdU washout during PS formation. As documented, the rapidly proliferating progenitor cells lose the BrdU label, whereas the slow-dividing stem cells retain BrdU (Hu et al. 2017). These PS cultures contained either vehicle (0.1% ethanol), 2.5, or 25 nM BPA during BrdU washout phase ($n=4$ /treatment). PS were harvested by 1 U/ml dispase digestion for 15 min and allowed to attach to chamber slides during overnight culture in ProstaLife™ medium. Spheres were fixed in ice-cold acetone/methanol (1:1) for 1 h and immunostained using mouse antihuman BrdU antibody 1:200 (Sigma-Aldrich) followed by secondary antibody goat antimouse Alexa Fluor® 488 (Invitrogen) with DAPI counterstain (Vector Laboratories). BrdU+ label-retaining cells were identified and counted in >100 spheres using fluorescent microscopy (Zeiss Axioskop 20).

Data Handling and Statistical Analysis

The primary de-identified data from all prostate studies were submitted to the Chemical Effects in Biological Systems (CEBS) database maintained by the NIEHS for secure storage as previously described (Heindel et al. 2015). Once the prostate studies were finished, the data were reviewed for completeness and locked down in CEBS, such that data could not be altered (read-only format). After primary data from all CLARITY-BPA Consortium studies were archived and quality review performed by the decoding team, the verified decoding information was shared with the PI (GSP) to permit the initial review of the findings and subsequent statistical analysis.

Body-weight data in each experimental group and between groups were compared by ANOVA followed by Tukey-Kramer multiple post hoc tests to determine significance. For histopathology studies, an initial power analysis determined that 18 animals/treatment were required to detect a significant increase in lesion incidence by BPA over background with 80% power at 0.05 significance based on previous studies by our laboratory (Ho et al. 2006). However, this number was not achieved in any of the experimental groups because only lower numbers of tissues were made available by the NCTR. For Set 3, where animals were unevenly distributed across treatment groups, the 25 and 2500 BPA μ g/kg-BW treatment groups were very small ($n=4$ each), and their data did not pass normality testing and were therefore omitted from further statistical analysis. One-way ANOVA followed by Dunnett's post hoc multiple comparison tests were used to determine differences in lesion incidence and severity scores (expressed as mean \pm SEM) between treatment groups. Tumor incidence and multiplicity were analyzed using the Fisher Exact test with Bonferroni correction for multiple comparisons.

For the stem/progenitor cell studies, the data for PS number and size were analyzed using Welch's one-way ANOVA followed by Games-Howell multiple comparisons post hoc tests due to unequal sample sizes and variances between groups. The PS gene expression data, which had equal variances, was analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparison post hoc tests to determine significance. Values are expressed as mean \pm SEM and $P < 0.05$ was considered significant.

Results

Histopathology of Rat Prostates at One Year of Age

Although the mean body weights at one year did not change within each treatment group in comparison with respective controls, the body weights in rats from Set 3 (stop-dose BPA, adult T + E) were lower in comparison with body weights in similarly treated rats from Set 2 (stop-dose BPA), with significance reached in the vehicle controls, EE, and the 250- μ g BPA/kg BW rats (Table S2). This phenomenon has been previously reported for long-term T + E exposure (Bosland et al. 1995; Prins et al. 2017). The prostatic complex, including the ventral, dorsal, and lateral lobes and the periurethral prostatic ducts of the three separate sets of rats exposed to BPA were assessed for prostatic lesions at one year of age. Set 1 comprised rats continuously exposed to different BPA doses from G6 throughout life. Minimal histopathologic lesions were observed in the prostatic tissues in the ventral lobe and the prostatic ductal regions and no pathology was identified in the dorsal lobes in control rats or any EE or BPA treatment groups (Table S3). The lateral lobes exhibited marked inflammatory lesions and associated reactive epithelial hyperplasia in the vehicle controls and across all groups treated with EE and BPA with no significant differences in incidence and severity noted between experimental groups as a function of BPA exposure (Table S3). Set 2 rats that were treated with vehicle, EE, or one of five doses of BPA from G6 through PND 21 likewise exhibited minimal and no pathology in the ventral and dorsal lobes, respectively, in

any treatment group (Table S4). A high incidence of inflammation and resultant reactive epithelial hyperplasia were observed in the lateral prostates of all Set 2 rats treated with vehicle, EE, or 5 doses of BPA, again with no statistically significant differences in incidence and severity noted between experimental groups as a function of BPA exposure. In addition, a 10–50% incidence of PIN was identified in the periurethral prostatic ducts of all treatment groups in Set 2 with no statistically significant differences in comparison with vehicle controls. It was noted that 6 of 10 vehicle-treated rats in Set 2 were housed in the same room with a separate set of rats treated daily with 250,000 µg BPA/kg-BW, either during gestation through PND 82 or post weaning through PND 23–57. To address the possibility of cross-contamination with BPA under these co-housing conditions, a proposed explanation for measured BPA in some control rats of the pre-CLARITY study (Churchwell et al. 2014), the co-housed control rats were excluded in a separate analysis that assessed only vehicle controls of the four nonexposed rats (Table S4). Similar results were noted between the two separate vehicle control groups (one with $n = 10$ and one with $n = 4$) with regard to lateral lobe inflammation, reactive hyperplasia, and prostatic duct PIN lesions.

Set 3 rats were treated with vehicle, EE, or one of five doses of BPA from G6 to PND 21 and T + E implants at PND 90 to drive prostatic hormonal carcinogenesis. There was 20% mortality in Set 3 rats as they were aged to 1 y, an occurrence not previously observed in our laboratories. This occurrence might indicate chronic stress, and/or the development of pituitary tumors due to E₂ treatment as previously observed (Bosland et al. 1995), although this was not measured herein. As in our prior reports using a T + E challenge (Ho et al. 2006; Prins et al. 2017), significant differences in prostatic pathology were observed in the lateral lobes of rats treated with BPA in comparison with controls that received only adult T + E (Table 1). The lateral prostates exhibited chronic inflammation and reactive epithelial hyperplasia in vehicle controls, EE-treated, and BPA-exposed rats at 100% incidence with no difference in severity scores between treatment groups. The incidence of T + E driven PIN lesions in the lateral lobes was also similar between controls and all BPA-treated groups. In contrast, developmental exposure to EE or 2.5, 250, or 25,000 µg BPA/kg-BW increased the PIN severity scores to high-grade PIN with the highest severity score (3.0 ± 0.4 ; $P < 0.01$) observed at the lowest BPA dose (2.5 µg/kg-BW) in comparison with vehicle control low-grade PIN (score = 1.3 ± 0.2) (Figure 1A; Table 1). Due to severe underpowering ($n = 4$) and non-normality of data, the 25 and 2500 µg BPA/kg-BW treatment groups were not statistically analyzed (described in Methods).

Adenocarcinoma was observed in the periurethral dorsolateral prostatic (DLP) ducts in all T + E treatment groups at 25–50% incidence rates with no statistically significant differences noted between BPA treatments vs. vehicle controls (Table 1). However, there was a four-fold increase in tumor multiplicity (tumor bearers only) in the lowest BPA-exposure group (2.5 µg/kg-BW); the difference was statistically significant in comparison with vehicle controls and EE-treated rats ($P < 0.001$) and the higher-dose BPA groups ($P < 0.01$) (Figure 1B–F). Although the tumor multiplicity in 250 to 25,000 BPA/kg-BW groups trended higher than the vehicle controls, the differences were not statistically significant. Periurethral ducts of the anterior prostate lobe (a.k.a. coagulating gland) also exhibited adenocarcinoma as a function of the T + E treatments, but there was no difference in incidence (ranging from 50–89%) or tumor multiplicity in these structures in the groups exposed to developmental EE or BPA exposure in comparison with vehicle control rats (Table 1).

Stem and Progenitor Cell Analysis

DLP stem and daughter progenitor cell quantity and lineage commitment were examined in 6-month-old rats treated daily for 6 months with vehicle, EE, or BPA at 2.5, 25, or 250 µg/kg-BW

using a PS-based assay as depicted in Figure 2A. Under these conditions, only stem-like epithelial cells are capable of surviving and undergoing asymmetric cell division to yield daughter progenitors that rapidly amplify to form PS of progenitor cells entering lineage commitment (Hu et al. 2011). To ensure assessment of true stem cells, the spheroids were dispersed after 7 d and passaged through 2 more PS generations, all in the absence of BPA. Two tissue sets at the 25 µg BPA/kg-BW dose did not yield results due to a laboratory technical problem, thus reducing that N number to 3. There was a significant increase in the total PS number (spheres > 40 µm size), a measure of the tissue stem cell number, in DLPs of rats exposed to EE or 2.5 µg BPA/kg-BW in comparison with vehicle controls (Figure 2B). There was no difference in PS number at 25 µg BPA/kg-BW vs. control prostates, and although numbers of PS were higher in the 250 µg BPA/kg-BW group, the difference was not statistically different from the controls. Next, we classified PS size, with 40–80 µm considered medium-sized and > 80 µm considered large, which reflects progenitor cell proliferative capacity. Although large-sized PS were minimal in P3 vehicle controls, *in vivo* exposure to EE or 25 µg or 250 µg BPA/kg-BW resulted in significantly more PS > 80 µm (Figure 2C), suggesting a permanent increase in progenitor cell proliferative capacity despite the absence of BPA during the 21-d culture period.

PS gene expression was next evaluated to assess lineage commitment of progenitor cells, which comprise the vast majority of cells within the spheroids. Expression of cytokeratins (CK) 5 and 8 were used to delineate basal and luminal progenitor lineages, respectively, and a set of stemness-associated genes previously characterized for the prostate (Prins et al. 2014) was assessed in parallel. As with increased progenitor cell proliferation, we observed significant differences in expression of gene sets in prostates exposed *in vivo* to 25 µg or 250 µg BPA/kg-BW, but not to the 2.5-µg dose (Figure 3A, B). A significant increase in basal progenitor markers (determined by alignment with CK5 expression) was observed in PS from rats exposed to EE and 25 µg BPA/kg-BW in comparison with vehicle controls (Figure 3A). A similar trend was noted for 250 µg BPA/kg-BW-exposed rats, although this trend was not statistically significant. Concurrently, *in vivo* exposure to EE, 25, and 250 µg BPA/kg-BW significantly suppressed luminal progenitor markers (determined by alignment with CK8) (Figure 3B). That stemness genes consistently aligned with either basal or luminal lineage markers suggests a lineage shift in progenitor populations as opposed to emergence of differentiated cells in the spheroids (Figure 3A, B). Similarly, exposure to EE resulted in significantly higher expression of CK5, *Sox2*, *Sox9*, and *Hoxb13* and significantly lower expression of CK8, *Tbx3* and *Trop2*, which is consistent with an estrogenic basis for the BPA-induced shift in lineage commitment to basal progenitors and away from luminal progenitors. There was no treatment-associated difference in chromogranin A gene expression, a neuroendocrine cell marker, in any exposure group in comparison with vehicle control prostates (Figure S1). Together, the findings suggest a dose-dependent effect of BPA, with the lowest dose (2.5 µg) influencing stem cell numbers and the higher doses of 25 µg or 250 µg BPA/kg-BW primarily targeting the progenitor cell population.

To extend our previous work with rats that found that early-life BPA exposures can modify later-life prostatic responses to E₂ (Ho et al. 2006; Prins et al. 2017), we assessed whether chronic BPA exposures *in vivo* might alter the sensitivity of the rat prostate stem and progenitor cells to E₂ treatment *in vitro*. Passage 3 PS were exposed to 1 nM E₂ for the 7-d culture period. Although *in vitro* E₂ had no effect on PS number from *in vivo* vehicle-treated DLPs, it resulted in increased PS numbers from DLPs exposed *in vivo* to EE or any BPA dose, although the difference was not statistically significant (Figure 4A). The gene expression profiles in the *in vitro*

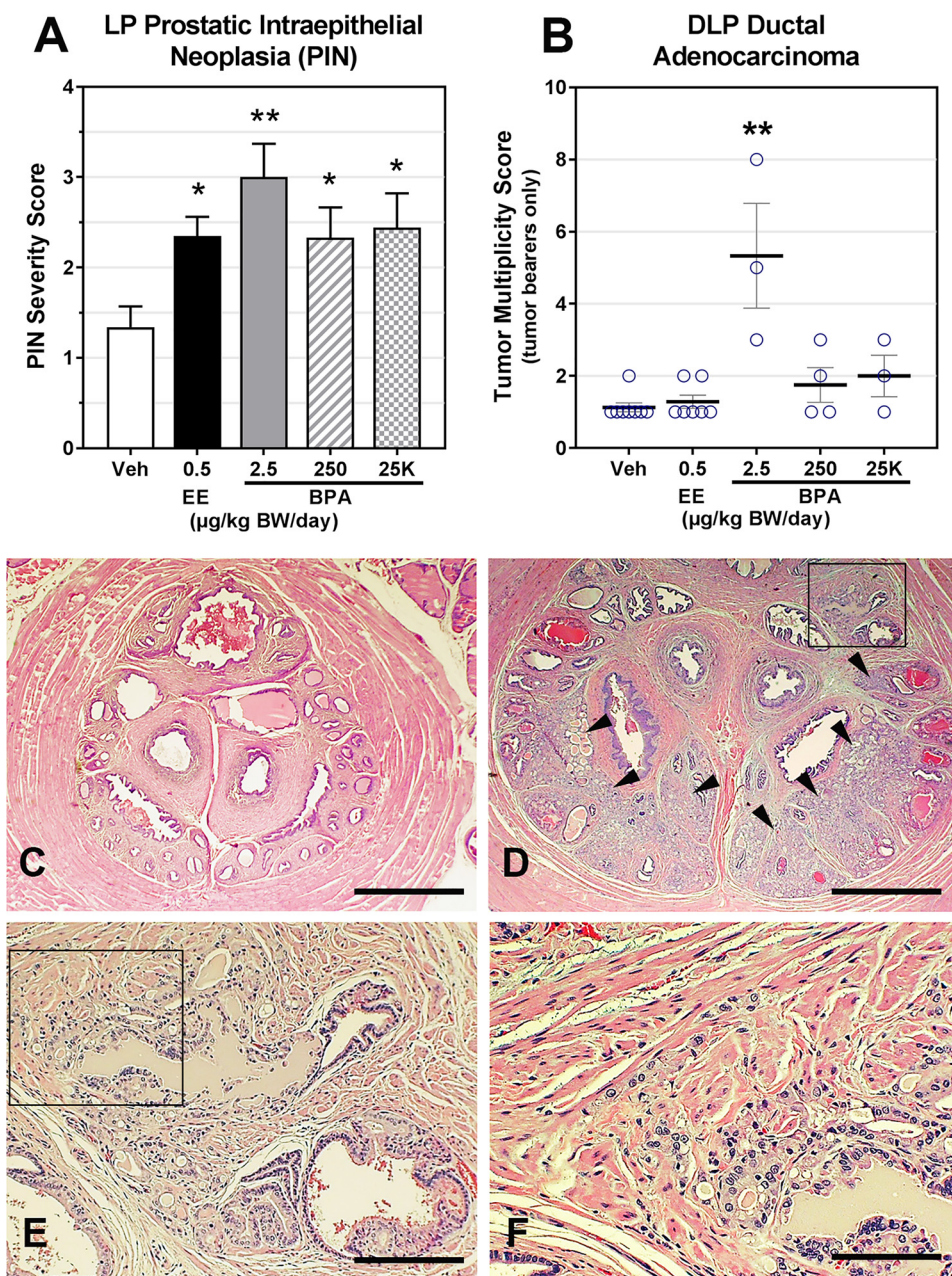


Figure 1. Pathology in prostates of Set 3 rats (stop-dose BPA, T + E at PND 90) at one year of age. Severity scores of lateral lobe PIN lesions (A) and Multiplicity of dorsolateral ductal adenocarcinoma (tumor bearers only); (B) in rats given vehicle (Veh), ethinyl estradiol (EE), or 2.5, 250, or 25,000 µg BPA/kg-body weight (BW) during development in comparison with vehicle controls. A: Bars represent the mean ± SEM. * $P < 0.05$, ** $P < 0.01$ vs. controls as determined by one-way ANOVA followed by Dunnett's multiple comparisons test. N # in () for each group: Veh (16), EE (17), and BPA at 2.5 (6), 250 (12), and 25,000 (9) µg/kg-BW. B: The black line represents the mean ± SEM, and each circle represents tumor number/rat. ** $P < 0.01$ vs. controls as determined by Fischer Exact test with Bonferroni correction for multiple comparisons. N # in () for each group: Veh (8), EE (7), and BPA at 2.5 (3), 250 (4), and 25,000 (3) µg/kg-BW. C–F: Representative images of the histology of the periurethral prostatic ducts. C: Periurethral area of a developmental vehicle control rat treated with adult T + E showing no tumors; bar = 1,000 µm. D: Periurethral area of a rat treated with 2.5 µg BPA/kg-BW developmentally and T + E in adulthood. Multiple adenocarcinomas (arrowheads) are observed in the dorsolateral prostate (DLP) ducts; a DLP tumor (in box) is shown in higher magnification in E; bar = 1,000 µm. E: Small adenocarcinoma originating from a prostatic duct in the periurethral area of a rat treated with 2.5 µg BPA/kg-BW plus adult T + E; bar = 200 µm. F: Higher power image of the adenocarcinoma in the panel E inset showing strands and nests of cancer cells invading the surrounding stroma; bar = 100 µm.

E₂-exposed PS exhibited trends similar to those observed in the absence of hormone, with higher expression of basal cell lineage markers and lower expression of luminal cell lineage markers in DLPs from rats exposed *in vivo* to EE, 25, or 250 µg BPA/kg-BW (Figure 4B, C). Noted differences were that expression of basal (*Hoxb13*) and luminal (CK8 and *Tbx3*) lineage markers in rats exposed *in vivo* to 2.5 µg BPA/kg-BW and *in vitro* to 1 nM E₂

were higher in comparison with vehicle controls. The lack of further modifications of PS properties with *in vitro* E₂ treatment may be related to the limited exposure time of 7 d. The overall effects of *in vivo* BPA exposure on rat prostate stem and progenitor cell populations are schematized in Figure 3C.

Our laboratory previously found that *in vitro* BPA exposures increased the number of PS cultured from human prostate primary

Table 1. Prostate histopathology at one year in Set 3 rats: Stop-dose treatment from G6 to PND21, T + E implants at PND 90.

| $\mu\text{g/kg BW/day}$ $n = \text{final\#}^a/\text{start\#}^b$ | Lateral prostate | | | | | | DLP Ducts | | AP Ducts | |
|--------------------------------------------------------------------|------------------|---------------|----------------------|---------------|---------------|--------------------|----------------|---------------------------------|----------------|---------------------------------|
| | Inflammation | | Reactive Hyperplasia | | PIN | | Adenocarcinoma | | Adenocarcinoma | |
| | Incidence (%) | Score | Incidence (%) | Score | Incidence (%) | Score | Incidence (%) | Tumor Multiplicity ^c | Incidence (%) | Tumor Multiplicity ^c |
| Vehicle $n = 16/19$ | 16/16 (100) | 2.9 ± 0.2 | 16/16 (100) | 2.4 ± 0.1 | 14/16 (88) | 1.3 ± 0.2 | 8/16 (50) | 1.1 ± 0.1 | 12/16 (75) | 1.7 ± 0.5 |
| 0.5 EE $n = 17/20$ | 17/17 (100) | 2.9 ± 0.2 | 17/17 (100) | 2.4 ± 0.1 | 17/17 (100) | $2.4 \pm 0.2^*$ | 7/17 (41) | 1.3 ± 0.2 | 15/17 (88) | 1.6 ± 0.5 |
| 2.5 BPA $n = 6/8$ | 6/6 (100) | 3.2 ± 0.3 | 6/6 (100) | 2.8 ± 0.2 | 6/6 (100) | $3.0 \pm 0.4^{**}$ | 3/6 (50) | $5.3 \pm 1.5^{**\dagger}$ | 4/6 (67) | 1.8 ± 0.5 |
| 25 BPA $n = 4/5$ | 4/4 (100) | | 4/4 (100) | | 2/4 (50) | | 1/4 (25) | | 3/4 (75) | |
| 250 BPA $n = 12/14$ | 12/12 (100) | 2.8 ± 0.1 | 12/12 (100) | 2.5 ± 0.2 | 11/12 (92) | $2.3 \pm 0.3^*$ | 4/12 (25) | 1.8 ± 0.5 | 8/12 (67) | 1.3 ± 0.5 |
| 2,500 BPA $n = 4/7$ | 4/4 (100) | | 4/4 (100) | | 4/4 (100) | | 1/4 (25) | | 2/4 (50) | |
| 25,000 BPA $n = 9/15$ | 9/9 (100) | 2.9 ± 0.2 | 9/9 (100) | 2.4 ± 0.2 | 9/9 (100) | $2.4 \pm 0.4^*$ | 3/9 (34) | 2.0 ± 0.6 | 8/9 (89) | 1.5 ± 0.5 |

Note: Due to severe underpowering ($n = 4$) and non-normality of data, the 25 and 2500 $\mu\text{g BPA/kg-BW}$ treatment groups were not statistically analyzed (described in main text in Methods). BPA, bisphenol A; BW, body weight; DLP ducts, dorsolateral prostatic ducts; AP ducts, anterior prostate (coagulating glands) ducts; PIN, prostatic intraepithelial neoplasia (dysplasia).

^{*} $P < 0.01$ vs. vehicle; ^{*} $P < 0.05$ vs. vehicle.

[†] $P < 0.05$ vs. EE (One-way ANOVA with Dunnett's multiple comparison tests).

^afinal count at autopsy.

^bstarting count in each treatment group.

^cMultiplicity assessed in tumor bearers only.

epithelial cells (Prins et al. 2014). More recently, we developed a BrdU label-retaining assay that permits direct evaluation of stem cell number within each PS and showed that this assay can be used to assess stem cell self-renewal activity (Hu et al. 2017). We

therefore applied this assay in the current study and directly tested whether exposure to BPA could alter the stem cell number within human PS. Exposure to 2.5 nM BPA for 7 d resulted in significantly higher numbers of BrdU + label-retaining stem cells per PS

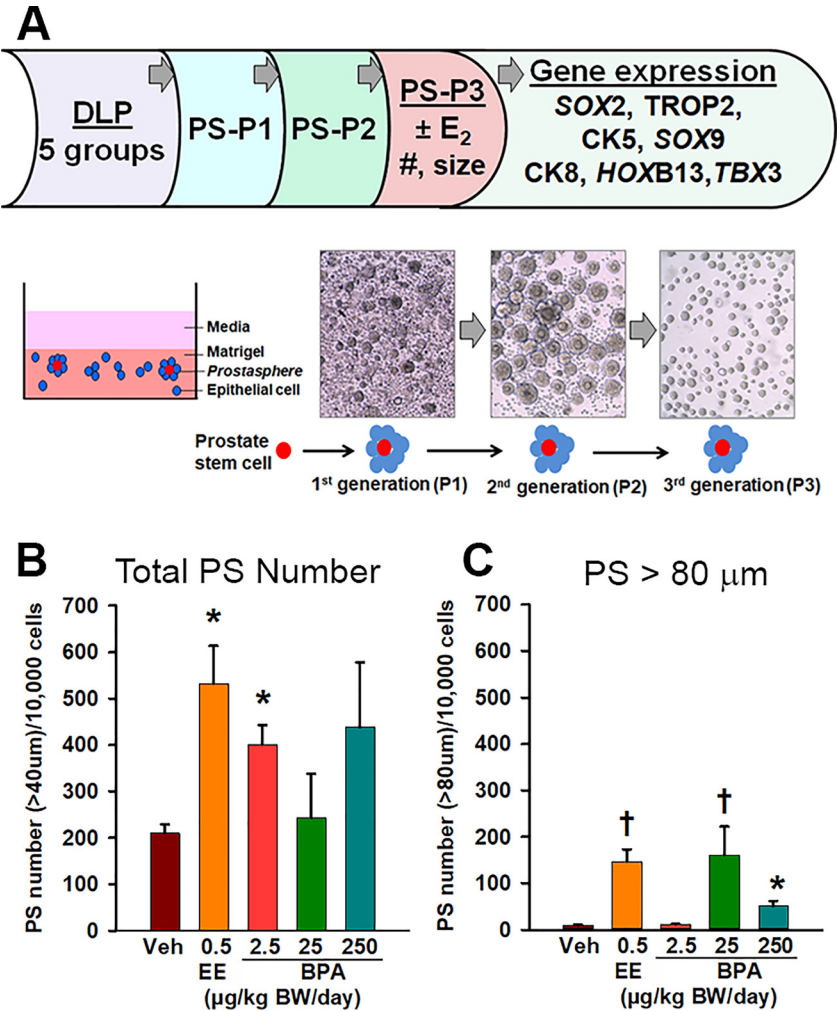


Figure 2. A: Experimental design for stem cell assessment in 6 mo old rat dorsolateral prostates (DLP) following daily gavage with vehicle (Veh), ethinyl estradiol (EE) or 2.5, 25, or 250 $\mu\text{g BPA/kg-body weight (BW)}$ (see Methods for details). B: Total prostatesphere (PS) cultures from the 5 treatment groups. $^*P < 0.02$ vs. vehicle as determined using Welch's one-way ANOVA followed by Games-Howell multiple comparisons tests. C: Measurement of large sized P3-PS (>80 μm) from DLPs exposed *in vivo* to Veh, EE, or 2.5, 25, or 250 $\mu\text{g BPA/kg-BW}$. All graphs represent mean \pm SEM. $^{\dagger}P < 0.01$ vs. vehicle, $^*P < 0.02$ vs. vehicle as determined by Welch's one-way followed by Games-Howell multiple comparisons test. N # in () for each group: vehicle (4), EE (5), and BPA at 2.5 (5), 25 (3), and 250 (5) $\mu\text{g/kg-BW}$.

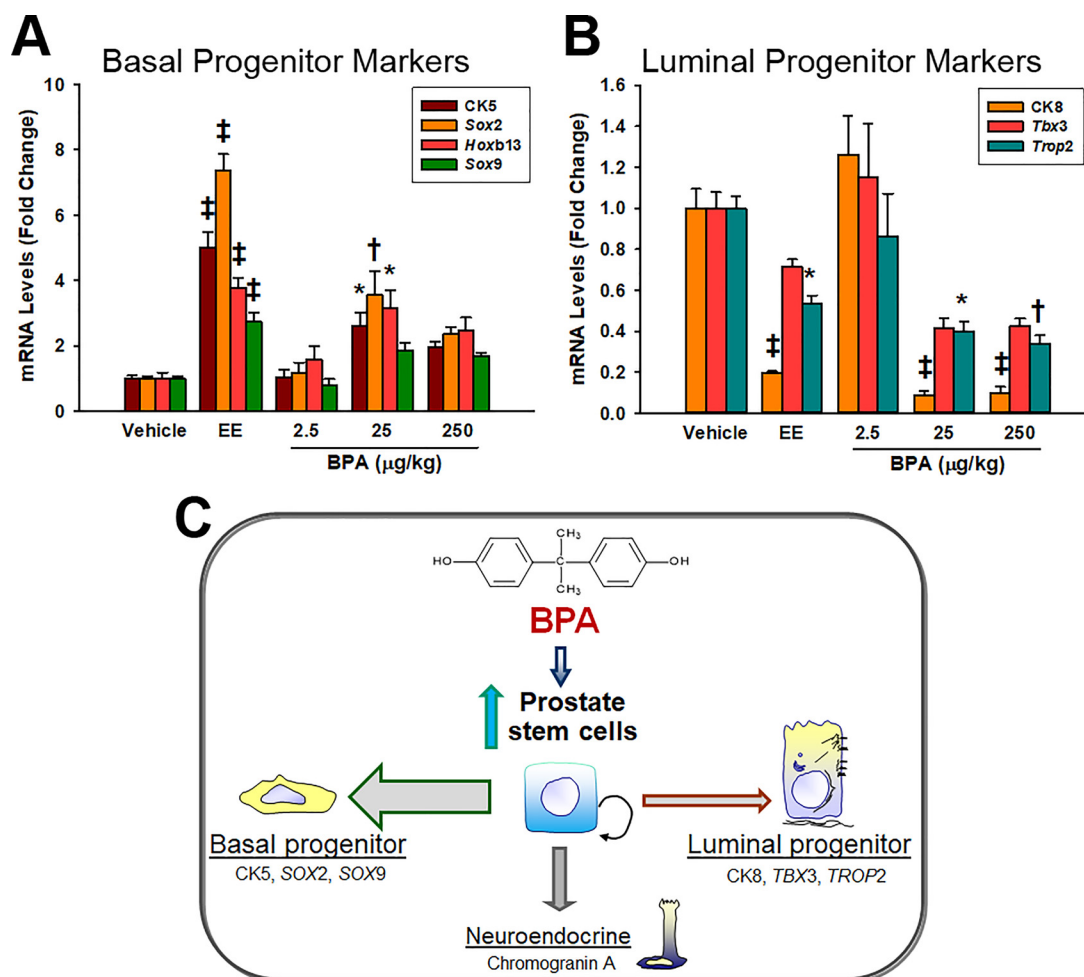


Figure 3. Gene expression in passage 3 prostaspheres (PS) as determined by qRT-PCR from the 5 treatment groups. Expression levels of basal progenitor markers (relative to CK5 expression) (A) or luminal progenitor markers (relative to CK8 expression) (B) in PS from rats exposed to Veh, ethinyl estradiol (EE), or 2.5, 25, or 250 μg BPA/kg-body weight (BW). The N# for each group are the same as in Figure 2. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ vs. vehicle as determined by ANOVA followed by Tukey-Kramer multiple comparisons test. All graphs represent mean ± SEM. C: A schematic representation of the effects of *in vivo* BPA exposure on prostate stem cell numbers and lineage commitment of progenitor cells. Chronic BPA exposures at the 2.5 μg dose increase stem cell numbers, whereas 25 μg or 250 μg BPA/kg BW exposures favor a basal progenitor lineage of stem cell progeny implicating a reprogramming of prostate stem cell population by BPA in a dose-dependent manner.

suggesting direct stimulation of stem cell symmetric self-renewal (Figure 5). In contrast, exposure to a 10-fold higher level of BPA resulted in significantly fewer BrdU+ stem cells per PS, implicating a brake on their symmetric self-renewal activity at higher doses (Figure 5).

Discussion

The present findings confirm previous reports (Ho et al. 2006; Prins et al. 2011; 2014; 2017; Wong et al. 2015) that developmental BPA exposures sensitize the prostate to later-life E₂-driven carcinogenesis, an apical adverse outcome. Specifically, in comparison with vehicle controls, perinatal exposure of rats to BPA at low (2.5 μg/kg-BW), medium (250 μg/kg-BW) and high (25 mg/kg-BW) doses resulted in more severe PIN lesions, shifting from low-grade PIN in controls to HG-PIN with the highest PIN severity score observed at the lowest BPA dose tested. Of note, in humans HG-PIN is the precursor lesion to PCa, whereas LG-PIN is not considered clinically relevant. Importantly, the 2.5 μg BPA/kg-BW exposure led to a four-fold higher adenocarcinoma multiplicity in the DLP ducts, an effect not seen at higher BPA doses. This finding parallels that in the

CLARITY-BPA Core studies (NTP 2018), which reported a significant increase in DLP cellular lymphocytic infiltration at one year in 2.5 μg BPA/kg-BW rats, an effect also not seen at higher doses. A strength of this study is that data collection and analysis were conducted in a manner blinded to treatment group, and all animals were handled at the U.S. FDA facility under GLP guidelines. This approach was taken to reduce the likelihood that bias might be inadvertently introduced during tissue analysis and thereby increases confidence in the evidence reported herein supporting our hypothesis that developmental BPA exposures prime the prostate for increased susceptibility to later-life cancers induced by natural hormones.

A number of previously published CLARITY-BPA studies detected the greatest and sometimes the only observed effects at 2.5 μg BPA/kg-BW, the lowest dose examined, providing evidence for unique responses to low-dose BPA at levels relevant to human exposures (Arambula et al. 2016; Gear et al. 2017; Patel et al. 2017). Importantly, an elevated incidence of mammary gland adenocarcinoma was seen only at 2.5 μg BPA/kg-BW in the female stop-dose group of the CLARITY-BPA Core studies (NTP 2018). Taken together, the observation of higher mammary adenocarcinoma incidence and DLP ductal adenocarcinoma multiplicity

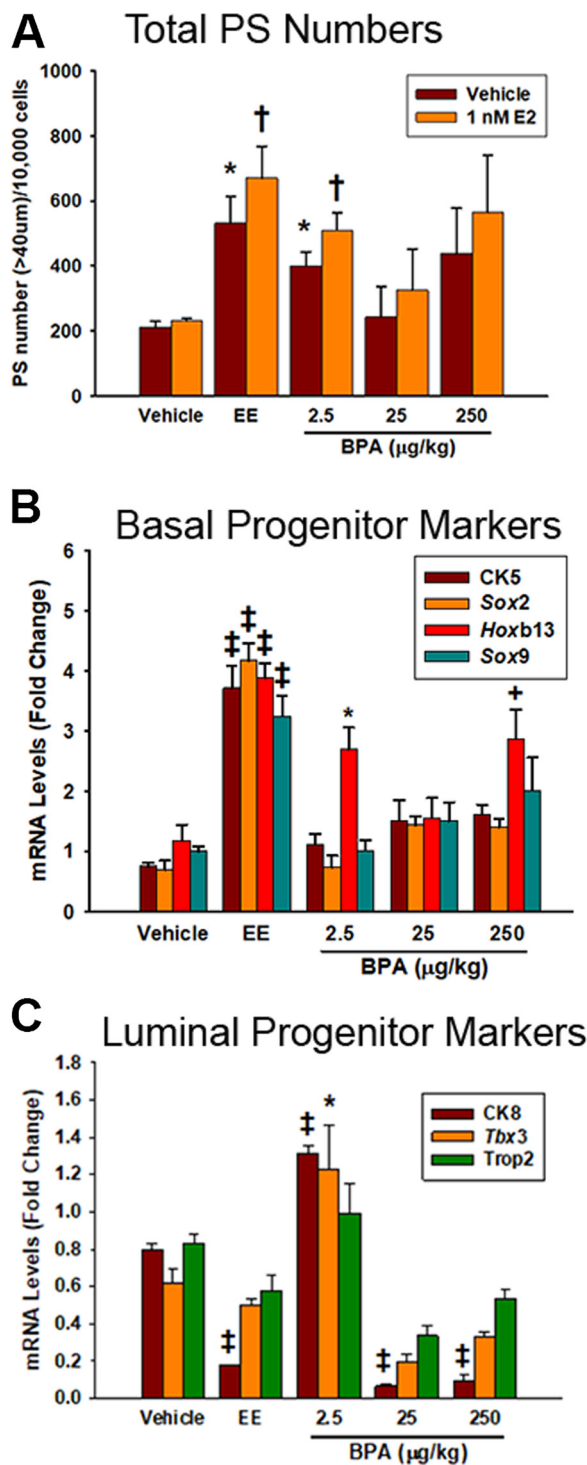


Figure 4. Total PS numbers and gene expression patterns in DLP-generated prostaspheres (PS) exposed *in vitro* to 1 nM estradiol-17 β (E₂) during passage 3 of spheroid culture. **A:** Total PS numbers following 7 d of exposure to E₂ to PS grown from rats exposed *in vivo* to vehicle (Veh), ethinyl estradiol (EE), or 2.5, 25, or 250 μ g BPA/kg-body weight (BW). * P < 0.02 vs. vehicle-control group; † P < 0.005 vs. Vehicle + 1 nM E₂ by one-way ANOVA followed by Games-Howell multiple comparisons test. **B** and **C:** Gene expression in PS from rats exposed *in vivo* to Veh, ethinyl estradiol (EE), or 2.5, 25, or 250 μ g BPA/kg-body weight (BW) with exposure to E₂ *in vitro* for that last 7 d of culture. **B:** Basal progenitor cell marker expression. **C:** Luminal progenitor cell marker expression. All graphs represent mean \pm SEM. The N # for each group are the same as in Figure 2. * P < 0.05, + P < 0.01, † P < 0.001 vs. vehicle as determined by one-way ANOVA followed by Tukey-Kramer multiple comparisons test.

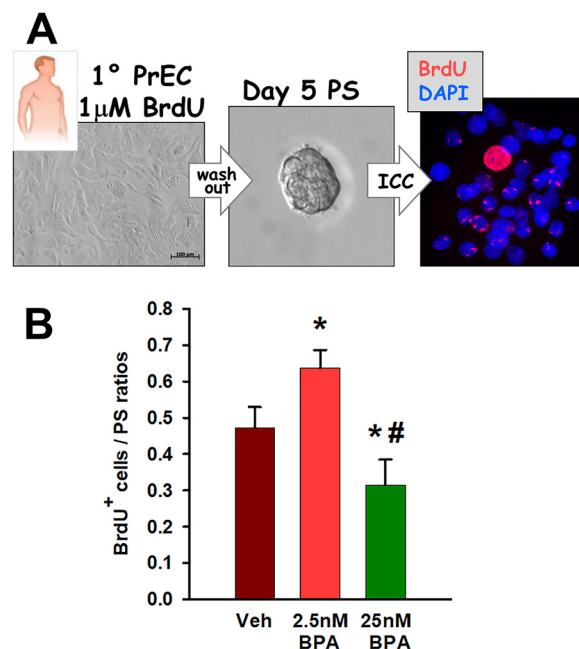


Figure 5. Human prostasphere stem cell response to bisphenol A (BPA) exposure *in vitro*. **A:** Schematic representation of the BrdU+ label retaining assay. Donor human prostaspheres are collected, and epithelial cells place in primary culture (1° PrEC) with 1 μ M BrdU for 10 d. Cells are transferred to 3D culture without BrdU for 5 d to form spheroids as described in Methods. Rapidly dividing progenitor cells washout BrdU label, and the primary stem cells remain relatively quiescent after initial symmetrical or asymmetrical self-renewal to form daughter progenitors, thus retaining the BrdU label long term. Immunohistochemistry for BrdU identifies the stem cell within spheroids which is quantified/PS. **B:** BrdU+ label-retaining stem cell numbers in human PS following *in vitro* exposure to 2.5 or 25 nM BPA. All graphs represent mean \pm SEM. * P < 0.05 vs. vehicle control; # P < 0.05 vs. 2.5 nM BPA as determined by ANOVA followed by Tukey-Kramer multiple comparisons test.

identified solely with perinatal exposure to 2.5 μ g BPA/kg-BW indicates that maximal carcinogenic BPA responses may uniquely occur with developmental-specific exposure at BPA doses well below those typically studied and far below the current NOAEL. This finding is consistent with earlier predictions by Soto and colleagues (Muñoz-de-Toro et al. 2005).

The current studies also confirm our previous observations (Ho et al. 2006; Prins et al. 2017) that developmental exposure to BPA alone at any dose is insufficient to induce histopathologic prostatic lesions and expand this research to show that continuous exposure from gestation through aging is likewise insufficient in driving prostate disease. Similar conclusions were drawn by the CLARITY-BPA Core study for nonneoplastic lesions in the dorsolateral and ventral lobes and neoplastic lesions in the ventral prostate in continuous dose and stop-dose animals one or two years of age (NTP 2018). As noted above, low-dose perinatal BPA treatment alone was sufficient for increasing mammary cancer incidence, suggesting a far greater carcinogenic sensitivity to BPA in that female target organ.

A brief discussion of the rat as a model for prostate cancer is warranted to place the present CLARITY-BPA findings in proper context. Unlike men, who develop prostate adenocarcinoma at high rates with aging (Zhou et al. 2016), most rat strains, including the Sprague-Dawley, do not spontaneously develop prostate cancer, highlighting fundamental biological differences in the carcinogenic process in the prostate and differences in risk-conferring exposures between the two species. The use of rats as a model for prostate carcinogenesis requires either potent chemical carcinogens or extended

exposure to natural sex steroids with high receptor affinity (Bosland 1996; Shirai et al. 2000). In this context, it is not surprising that BPA exposure alone is not a carcinogen in the rat prostate because it has reduced affinity, relative to E₂, for nuclear ERs in prostate cells (Prins et al. 2014). However, absence of such an effect does not ensure that BPA exposure is safe for the human prostate. Of the compounds used to induce prostate cancers in rats, extended exposure to testosterone at physiological levels with two-fold elevated E₂ is physiologically relevant because E₂ levels rise in aging men (Vermeulen et al. 2002). As such, developmental BPA exposure combined with adult T + E, as used herein, is the most relevant experimental regimen for testing BPA effects on prostate carcinogenesis in a rat model with potential for direct applicability to humans.

A limitation of the present experiments was the very low number of animals in the 25 and 2500 µg/kg-BW groups given adult T + E (*n* = 4) that did not permit their inclusion in statistical analysis. The number of animals in the 2.5 µg BPA/kg-BW dose group was also low (*n* = 6); however, the effect size was robust in the statistical analysis despite the low numbers. The underpowering of this study at the NCTR labs defies the U.S. FDA's own recommendations of 50 animals per group for carcinogenicity studies (Aungst and Twaroski 2009), which was the number used for the parallel CLARITY-BPA Core study (NTP 2018) and was outside the control of the authors despite repeated requests for more animals. Notwithstanding this serious shortcoming, consistency of the results of the current study with previous findings by two separate laboratories (Prins et al. 2017; Wong et al. 2015) supports our conclusion that developmental BPA exposures increase PCa risk in the T + E rat model.

Several differences between the current pathology results and previously reported findings using a similar model deserves further discussion. First, few ventral or dorsal lobe lesions were noted in the current study using NCTP Sprague-Dawley rats, bred at the U.S. FDA facility for >30 years, which contrasts with our previous dose-response study using Harlan (now Envigo) Sprague-Dawley rats where inverted U-shaped dose-response curves were observed in PIN severity in those prostate regions (Prins et al. 2017). Further, the incidence of lateral lobe PIN and DLP ductal adenocarcinomas was not affected by perinatal BPA or EE exposure with adult T + E treatment in the present experiments, whereas our previous findings found elevated PIN and carcinoma incidences in the lateral lobe at 7 months and one year, respectively, in rats treated neonatally with 10 µg BPA/kg-BW plus adult T + E (Prins et al. 2017). These divergent findings and differences in inflammation likely result from multiple variations in experimental designs between studies, including differences in Sprague-Dawley rat substrains, diet compositions, exposure periods (G6 to PND 21 vs. PND 1, 3, 5 used previously), and exposure routes (daily gavage vs. s.c. oil depot used previously), as well as the lack of T + E tube replacement every 8 wk in the present study as was done previously. It is noteworthy that chronic and high incidences of lateral prostate inflammation were found in all rats in the present studies, including control rats in Sets 1 and 2 with 80–100% penetrance, possibly related to housing conditions and treatment protocols, and elevated mortality that occurred in Set 3 T + E -treated rats, phenomena not observed in our prior work with Sprague-Dawley rats. Despite these divergent design details that may account for differences noted in histopathology experimental findings, the overall conclusions regarding BPA effects on the prostate remain the same between studies.

Increased carcinogenic risk with BPA exposures as noted herein and previously may be underpinned, in part, by alterations in the stem/progenitor cell homeostasis with low-dose BPA (2.5 µg/kg-BW) increasing DLP stem cell numbers, whereas higher doses augment progenitor cell proliferation and modify lineage commitment. With the additional *in vitro* exposure to E₂ for the last 7 d of culture, spheroids grown from rats exposed

in vivo to 2.5 µg BPA/kg-BW also showed elevated expression of luminal progenitor markers as well as *Hoxb13*, suggesting dysregulation of normal lineage commitment at the lowest BPA dose. It is important to note that elevated carcinogenic susceptibility to adult E₂ occurred herein with developmental BPA exposures, whereas the present stem/progenitor cell analyses followed chronic BPA exposure; thus, direct cause-effect cannot be established. Nonetheless, it is conceivable that chronic BPA exposures would also increase carcinogenic susceptibility in the aging prostate. The stem/progenitor cell changes align with our previous work in rats that found permanent epigenetic alterations leading to upregulation of prostate *Sox2*, *Wnt10B* and other key stemness genes by low-dose BPA, observable at PND 90 (Cheong et al. 2016; Prins et al. 2017). Of critical importance, epigenetic mechanisms are central to maintaining stem cell identity (Mikkelsen et al. 2007), and disruptions in their epigenome may give rise to populations that are poised for neoplastic events by carcinogenic promoters, such as estrogen. Several recent reports have similarly identified stem cell alterations in multiple tissues exposed to BPA pointing towards stem cells as a common BPA target (Clément et al. 2017; Eladak et al. 2018; Lillo et al. 2017; Okada et al. 2010; Prins et al. 2015; Weng et al. 2010; Yang et al. 2013). Together, these results provide direct evidence that BPA exposures modify prostate stem cell self-renewal capability, that the effects are dose-specific and that the results in rats may be directly relevant to the human prostate.

That the stem/progenitor cell responses seen over the 2.5 to 250 µg BPA/kg-BW range were also observed in the EE-exposed prostates suggests that the BPA effects may be mediated through ER pathways. The dose-specific responses noted over the 100-fold BPA dose range could be due to differential engagement of ER populations, both in type (ERα, ERβ, GPER) and location (membrane and nuclear), at different doses as previously reported in both stem/progenitor cells (Hu et al. 2011; Okada et al. 2010; Prins et al. 2014) and differentiated cells (Alonso-Magdalena et al. 2012; Hu et al. 2011; Li et al. 2018; Okada et al. 2010; Prins et al. 2014; Wang et al. 2016; Watson et al. 2007). Chronic exposure to 2.5 µg BPA/kg-BW doubled the DLP spheroid numbers over three PS passages, which is considered a hallmark of a true stem cell population. Although long-term labeling of stem cells was not an option for the animal studies, experiments using the *in vitro* label-retaining assay in human PS demonstrated that low-dose BPA (2.5 nM) exposure significantly increased stem cell symmetrical self-renewal. This increase is highly relevant because cancer risk has been proposed to be strongly correlated with the number of normal stem cell divisions across multiple tissues, including the prostate (Tomasetti and Vogelstein 2015; Tomasetti et al. 2017). Because the 2.5 µg BPA/kg-BW dose is the same dose that led to significantly greater tumor multiplicity in DLP ducts, the present findings suggest that BPA reprogramming of stem cell numbers might contribute to increased susceptibility to E₂-induced prostate tumor formation.

Although the 25 and 250 µg *in vivo* BPA doses did not result in higher stem cell numbers, they markedly affected the daughter progenitor cell populations by increasing their proliferation and shifting lineage commitment to basal progenitors at the expense of the luminal population. Similarly, using human PS, the 10-fold higher BPA dose (25 nM) resulted in less symmetrical stem cell self-renewal and greater PS size which may reflect a shift to asymmetric division. The altered lineage commitment toward basal-cell progeny in the rat DLP is reminiscent of previous findings that consistently showed that brief early-life estrogenic exposures increased basal-cell numbers along with differentiation defects in luminal cells in the rat (Prins and Birch 1993) and mouse prostate lobes (Prins et al. 2001). This increase is noteworthy because

several studies have shown that tumor initiating cells for human PCa are localized to the basal-cell compartment (Goldstein et al. 2010; Taylor et al. 2012). Consequently, higher prostate basal-cell populations may stoichiometrically increase the opportunity for tumor initiation by secondary exposures, as observed herein.

In summary, the present CLARITY-BPA prostate study findings confirm that although developmental and chronic BPA exposures are not carcinogenic to the rat prostate, they enhance carcinogenic susceptibility to later-life estrogen exposures, with the greatest effects observed at the lowest BPA dose examined. Furthermore, chronic low-dose BPA exposure reprograms adult rat prostate stem cell homeostasis with marked increases in stem cell numbers and a shift in lineage commitment toward basal progenitor cells, which may underpin the increased carcinogenic risk with aging. Together, the results provide unbiased evidence that BPA exposures at human-relevant doses result in adverse effects on the rat prostate gland.

Acknowledgments

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